Development of genetic monitoring methods for genetic conservation units of forest trees in Europe. European Forest Genetic Resources Programme (EUFORGEN)

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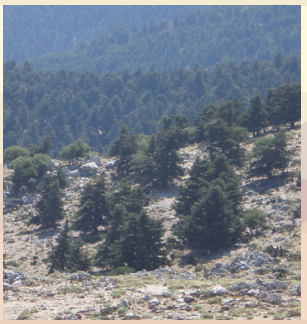

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European Forest Genetic Resources Programme (EUFORGEN) is an instrument of international cooperation promoting the conservation and appropriate use of forest genetic resources in Europe. It was established in 1994 to implement Strasbourg Resolution 2 adopted by the first Ministerial Conference of the FOREST EUROPE process, held in France in 1990. EUFORGEN also contributes to implementation of other FOREST EUROPE commitments on forest genetic resources and relevant decisions of the Convention on Biological Diversity (CBD). Furthermore, EUFORGEN contributes to the implementation of regional-level strategic priorities of the Global Plan of Action for the Conservation, Sustainable Use and Development of Forest Genetic Resources (GPA-FGR), adopted by the FAO Conference in 2013. The Programme brings together experts from its member countries to exchange information and experiences, analyse relevant policies and practices, and develop science-based strategies, tools and methods for better management of forest genetic resources. Furthermore, EUFORGEN provides inputs, as needed, to European and global assessments and serves as a platform for developing and implementing European projects. EUFORGEN is funded by the member countries and its activities are mainly carried out through working groups and workshops. The EUFORGEN Steering Committee is composed of National Coordinators nominated by the member countries. The EUFORGEN Secretariat is hosted by Bioversity International. Further information on EUFORGEN can be found at www.euforgen.org.

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PREFACE

Several schemes have been set up or proposed for monitoring the health and biodiversity of European forests during the past 30 years. Unfortunately, none of the existing forest monitoring schemes collects data on genetic diversity, which is the engine driving adaptation of forests to climate change and maintaining biological diversity at species and ecosystem levels. Genetic monitoring, i.e. tracking of temporal changes in the genetic variation and structure of tree populations, is the only way to verify how well genetic diversity is maintained over time, and how this diversity is shaped by climate change and management practices.

At the global level, the first comprehensive genetic monitoring schemes for forest trees were proposed in the 1990s. Nevertheless, they were not incorporated into the various criteria and indicators (C&I) that were developed for assessing the sustainability of forest management in different parts of the world. The pan-European C&I for sustainable forest management, adopted by the FOREST EUROPE process in 1994, include genetic indicators. However, these indicators only track the amount of area managed for *in situ* and *ex situ* conservation of forest genetic resources, as well as seed production, in different countries. Therefore, the existing genetic indicators do not reveal the extent of genetic diversity maintained within the conserved tree populations in Europe.

This problem has been widely acknowledged and frequently discussed in many European countries. Subsequently, some countries have initiated pilot studies to test the feasibility of different genetic monitoring approaches. Several European research projects on forest genetic resources have also highlighted the problem and urged policy-makers to address the issue. At the pan-European level, the lack of genetic monitoring has also been discussed in the context of the European Forest Genetic Resources Programme (EUFORGEN). The programme was established in 1994 as a pan-European implementation mechanism of Strasbourg Resolution 2 (Conservation of Forest Genetic Resources), adopted by the first Ministerial Conference of the FOREST EUROPE process in 1990.

EUFORGEN started its activities with pilot networks on a few model tree species, and it gradually evolved into a collaborative platform focusing on broader groups

of tree species and, more recently, on thematic issues. During Phase III (2005–2009) of EUFORGEN, the Scattered Broadleaves Network developed a background document on genetic monitoring and recommended that such effort should focus on dynamic conservation units of forest trees, instead of trying to monitor changes in genetic diversity in all forests. Subsequently, the Steering Committee, consisting of representatives of all member countries, decided that EUFORGEN should continue its work on genetic monitoring during Phase IV (2010–2014). In 2010, the Steering Committee then requested a working group to review genetic monitoring methods and to propose options for creating a pan-European genetic monitoring system for the dynamic conservation units of forest trees.

This report presents the findings and recommendations of the EUFORGEN working group on genetic monitoring. The report was prepared by the working group members (the authors of this report), who organized their first meeting at Bioversity International in Maccarese, Italy, on 17–19 January 2012, and their second one at the School of Forestry (ETSI Montes) of the Technical University of Madrid (UPM) in Spain on 22–24 May 2012. An earlier version of this report was presented to a larger group of experts at the EUFORGEN workshop on conservation and monitoring of forest genetic resources that was organized in Järvenpää, Finland, on 18–20 September 2012 in collaboration with the Finnish Forest Research Institute (now Natural Resources Institute Finland). The inputs and comments from the workshop participants and other national experts contributing to the EUFORGEN work are gratefully acknowledged. The draft report was presented to the EUFORGEN Steering Committee for further review during its 8th meeting, held in Paris, France, on 27–28 November 2012. The Steering Committee endorsed the proposed approach for genetic monitoring, and decided that further development of a pan-European genetic monitoring scheme for forest trees should be based on Option 2 presented in that report. The Steering Committee also expressed its appreciation to the working group for the large amount of work done and for delivering the expected outputs, and requested the working group to finalize its report after carrying out some follow-up analyses. The working group then prepared a revised draft report and presented it to the Steering Committee at its 9th meeting, held in Tallinn, Estonia, on 3–5 December 2013. The Steering Committee provided some additional comments, endorsed the report and requested the working group to finalize it for printing.

CONTENTS

ACRONYMS USED IN THE TEXT

C&I	criteria and indicators
CBD	Convention on Biological Diversity
DBH	diameter at breast height
ddRAD	double digest restriction-associated DNA
EUFGIS	European Information System on Forest Genetic Resources
EVOLTREE	Evolution of Trees as Drivers of Terrestrial Biodiversity [project 2006–2010]
FGR	forest genetic resources
$(GD)^2$	Geo-referenced Database on Genetic Diversity
ICP Forests	International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests
IPCC	Intergovernmental Panel on Climate Change
NGR	next-generation sequencing
nSSR	nuclear microsatellites
RAD	restriction site-associated DNA
SNP	single nucleotide polymorphism
SSR	simple sequence repeat

EXECUTIVE SUMMARY

Genetic diversity is an essential element of tree species adaptation to climate change and other environmental changes. While several schemes have been set up during the past 30 years to monitor the health and biodiversity of forests in Europe, none of them expressly collects information about status and trends of genetic diversity. EUFORGEN has emphasized the importance of genetic monitoring as part of its previous activities and more recently, the Steering Committee established a working group to review genetic monitoring methods and to propose options for creating a pan-European genetic monitoring system for genetic conservation units of forest trees. This report presents the findings and recommendations of the working group.

The working group concluded that a system for genetic monitoring of the genetic conservation units would be an invaluable tool for conservation of forest genetic resources and for sustainable forest management. It recognised that the intent of genetic monitoring efforts is expanding, from the temporal assessment of genetic diversity and the processes that maintain it, to the evaluation and conservation of the adaptive potential of genetic diversity. This is important because it offers an early warning system that would increase the chances of implementing actions to reduce potentially harmful effects, especially under rapidly changing environmental conditions.

The working group assessed existing practices and as a result, it suggests specific approaches to:

- Identify regions for genetic monitoring
- Identify units for genetic monitoring within these regions
- Design genetic monitoring plots in the selected units
- Select indicators and verifiers for genetic monitoring

Implementation of these recommendations will result in a comprehensive and unified scheme, unique for Europe and of global significance.

Despite the increase in conservation of biodiversity since the coming into force of the Convention on Biological Diversity in late 1993, very few programmes exist to monitor status and trends of genetic diversity. In forestry, criteria and indicators have been agreed to decide whether forestry is sustainable, but these do not address

genetic diversity directly. Monitoring genetic diversity over time will permit estimates of demographic and genetic parameters, which in turn may indicate whether a population is undergoing adaptive change. Schemes to undertake such monitoring have been proposed since 1996, based on a selection of indicators that reflect the state of the population and verifiers or parameters to assess the indicators. Discussion on indicators and verifiers is ongoing and important; the group noted, however, that the current indicators used in Europe to monitor diversity – area managed for conservation and utilisation of forest tree genetic resources (*in situ* and *ex situ* genetic conservation) and area managed for seed production– are all effectively response indicators. That is, they reflect human activity to conserve genetic diversity. While they all appear to be showing an increase over time, they offer no information about the actual genetic diversity of the resources.

The working group considered indicators and verifiers in detail and sought to establish a minimum set of informative indicators that could be used across Europe, bearing in mind the ease of measuring verifiers, the technical expertise required, costs and the independence of indicators. Building on the existing schemes, the working group proposes two indicators – 1) selection and 2) genetic variation and mating system – assessed with a set of 10 or 11 verifiers. This approach would require demographic, genetic and genomic data, which could be produced at three different levels of completeness and, hence, cost, in particular (i) basic, (ii) standard and (iii) state of the art. The report contains details of indicative costs for the range of intensity of genetic monitoring, considering also likely advances in DNA-sequencing technologies.

The geographical regions selected for genetic monitoring should ideally be co-located with the regions selected for genetic conservation. The working group examined three different approaches for selecting the monitoring regions, akin to the approaches that underlie the selection of the regions for dynamic conservation units. In its suggestion for a pilot study, the working group detailed the criteria to be used for an expert assessment and mapped the results for *Fagus sylvatica* and *Populus nigra*.

The genetic monitoring units within the identified regions should be selected to include all ecological situations in which a given species occurs, also taking into account the existing information on genetic diversity and phylogeographic patterns (for example, to include units at the trailing edge of a species distribution range). The genetic monitoring units would be most efficiently selected from among units that are already being managed and assessed for other purposes. Size (in terms of number of individuals), ownership (public being preferred) and vulnerability to

other threats are also important criteria for selecting the monitoring units. Within the monitoring units, specific plots would be established for genetic monitoring to take advantage of the existing monitoring efforts, as this will reduce the need to record basic data for precise plot documentation.

The data and samples gathered as part of the genetic monitoring efforts need to be stored safely for decades. This will increase the value of genetic monitoring if they can be made available for further study and analysis in the future. The working group considered a variety of options and recommends that the EUFGIS database be adapted to record information on genetic diversity alongside other data recorded for each dynamic conservation unit. DNA samples could be stored as part of the EVOLTREE DNA Repository Centre, pending the development of analytical techniques that may provide additional useful information.

Another crucially important factor in pursuing a genetic monitoring programme is the commitment of countries and national agencies. The need for coordination and cooperation makes the development and implementation of a pan-European genetic monitoring scheme an essential component of EUFORGEN.

INTRODUCTION

Definition and importance of genetic monitoring

Almost 20 years ago, the United Nations' Convention on Biological Diversity (CBD) recognized conservation of genetic diversity as a key component of biodiversity conservation. Various actions have been carried out at national and international levels to implement the CBD commitments, but these have largely focused on habitat and species diversity and neglected genetic diversity (Laikre *et al.*, 2010). Furthermore, although Article 7 of the CBD also called for action to "monitor through sampling and other techniques the components of biological diversity" (CBD, 1993), very few international or national actions have been launched for monitoring genetic diversity or developing genetic indicators to collect information for designing conservation policies (Laikre, 2010).

Conservation of forest genetic resources (FGR) is one of the many goals of sustainable forest management. A number of different criteria and indicators (C&I) have been developed by regional processes to outline conditions that have to be met before forest management is considered sustainable. However, the existing C&I poorly address monitoring of genetic diversity for sustainable forest management (McKinnell, 2002).

Genetic monitoring includes two aspects: the assessment of the genetic status of a [forest tree] population and the temporal nature of the evaluation(s). Genetic monitoring has been defined as the quantification of temporal changes in population genetic variation and structure, generated by measurements of appropriate parameters (Aravanopoulos, 2011), or as the observation of the dynamics of transition from the present to the future genetic status of a forest stand (adapted from Konnert *et al.*, 2011). Hansen *et al.* (2012) and Schwartz *et al.*, (2007) have provided a more restrictive definition: analysis of molecular markers through time in order to estimate demographic and/or population genetic parameters, with the aim of inferring whether adaptive changes are occurring. Therefore, genetic monitoring focuses on a special, but integral, part of bio-monitoring, in the same sense that genetic conservation forms a special part of biological conservation (Aravanopoulos, 2011).

Historical development

At the global level, the first comprehensive genetic monitoring system for forest trees was proposed by Namkoong *et al*. (1996) based on four indicators and 18 verifiers (parameters) used to assess them. A slightly revised set of 14 demographic and nine genetic verifiers to assess changes in the status of these indicators were further developed by Namkoong *et al*. (2002). However, these verifiers are expensive and time-consuming to sample and measure as part of normal forestry operations, and the work requires a rather high level of scientific skills. For this reason, the system has not been adopted by any of the C&I processes promoting sustainable forest management.

Despite its practical limitations, the scheme of Namkoong *et al*. (1996) provides a useful conceptual framework for further development of genetic monitoring schemes, but several difficult questions remain. These include selection of species, characterization of genetic variation, threshold values of different verifiers, and evaluation of combined information from multiple indicators. All the above constitute critical information needed in order to reach clear conclusions on the success of genetic conservation (Boyle, 2000). It is extremely hard to develop a universal genetic monitoring system that can be applied systematically to genetic conservation units, protected areas and production forests. Furthermore, considering the challenges and constraints involved, a genetic monitoring system could be based on a representative sample of conservation units or managed forests, needed to draw conclusions on how well genetic diversity is maintained.

In Germany, a pilot genetic monitoring system was developed based on the indicators proposed by Namkoong *et al*. (1996) and it was recently tested by Konnert *et al*. (2011) using permanent monitoring plots. The establishment of specific plots for genetic monitoring makes it more feasible to sample and measure tree populations at different times and allows a better use of limited resources. The pilot field-testing confirmed that multiple indicators do not necessarily provide clear results regarding the long-term adaptive potential of the population under study.

Recently, Aravanopoulos (2011) proposed a simplified genetic monitoring system based on the earlier efforts on the topic, including the scheme of Namkoong *et al*. (1996). The development of this system was also discussed with the EUFORGEN Scattered Broadleaves Network. Aravanopoulos (2011) suggested that genetic monitoring should focus on the dynamic conservation units of forest trees and both keystone and rare/endangered species. The proposed genetic monitoring approach includes only three indicators, which are evaluated

based on three demographic and four genetic verifiers (Aravanopoulos, 2011). This is a useful contribution to make genetic monitoring more feasible, but it does not solve all the problems and needs to be tested.

In 2010, the EUFORGEN Steering Committee decided that the development of a pan-European approach for genetic monitoring of the genetic conservation units should be continued during Phase IV of the Programme (2010–2014). For this purpose, it established a working group on genetic monitoring and requested it to:

- Develop a synthesis of existing documents.
- Analyse the EUFGIS and other databases relevant for genetic monitoring purposes (e.g. ICP Forest).
- Develop recommendations for improving EUFGIS data standards for genetic monitoring.
- Present options for genetic monitoring methods, including defining time intervals for monitoring (per tree species group).
- Assess the cost of the options for genetic monitoring methods.
- Prepare a draft report.

The following chapters present the findings and recommendation of the working group on genetic monitoring.

STATE OF THE ART

Terminology (criteria, indicators, verifiers)

A number of basic terms have been used in the genetic monitoring of forest trees. Their definitions are presented below.

Criterion is a standard that a thing is judged by (without being a direct measure of performance), e.g. "functions and processes that preserve genetic variation are maintained" (after Boyle, 2000). A criterion will thus normally reflect a goal, or an objective. Namkoong *et al*. (2002) for example operated with one criterion: "conservation of the processes that maintain genetic variation". In current CBD terminology, a similar criterion has been referred to as a "headline indicator".

Indicator applies to any component or process of the forest ecosystem used to infer attributes of the sustainability of the resource, e.g. selection, or directional change in allele frequencies (after Boyle, 2000). Commonly, an indicator can be measured periodically to reflect change related to the objective for measuring the indicator.

Verifier implies parameter data, or information that enhances the specificity or the ease of assessment of an indicator, e.g. number of alleles (after Boyle, 2000). In common practical terms, the verifier is the measure of the indicator.

Genetic conservation unit refers to a forest stand or forest area, designated for dynamic genetic conservation. In the European context, a genetic conservation unit meets the pan-European minimum requirements for dynamic conservation units of forest trees (Koskela *et al*., 2013) and that are subsequently entered into the EUFGIS database (http://portal.eufgis.org).

Genetic monitoring unit is a dynamic conservation unit of forest trees that has been selected for a monitoring scheme according to a specified set of criteria.

Genetic monitoring plot is a delineated area within a genetic monitoring unit where monitoring observations and sampling take place. The size of the plot is determined by the required minimum number of individual trees at reproductive maturity and the presence of regeneration.

Types of indicators

The indicators suggested by Namkoong *et al*. (1996) and later papers, when assessed over time, reveal the status of the

genetic diversity and its structure (stable versus changing). These indicators are therefore referred to as state indicators. Under CBD, other types of indicators are also identified as well: pressure indicators are used to address the question of why we lose biodiversity; the impact is measured by benefit indicators; and response indicators are used to measure what we do to solve the anticipated problems and assess the sufficiency of action (UNEP/CBD/AHTEG, 2011). The discussion on indicators remains highly relevant and is ongoing. Criteria and indicators for sustainable forest management at the Pan-European level have been developed by the FOREST EUROPE process (previously the Ministerial Conference on the Protection of Forests in Europe, MCPFE) (http://www.foresteurope.org). As part of the preparation of the State of the World's Forest Genetic Resources report (FAO, 2014), a thematic study on indicators for forest genetic resources was developed (Graudal *et al*., 2014) and summarized as a review paper (Graudal *et al*., 2014). These papers discuss all types of indicators and their relevance at different levels, from the global down to the local. Four types of indicators were defined by Sparks *et al*. (2011):

- State indicators analyse the condition and status of aspects of biodiversity.
- Pressure indicators monitor the extent and intensity of the causes of biodiversity loss that responses aim to address.
- Response indicators measure the implementation of policies or actions to prevent or reduce biodiversity loss.
- Benefit indicators quantify the benefits that humans derive from biodiversity.

In this terminology, state indicators are the only direct measures of the status of diversity itself, and response indicators are reserved for human intervention. Assessing the state of the adaptive potential of forest tree genetic diversity also includes an assessment of genetic processes, which constitute the response of biodiversity itself (cf. Namkoong *et al*., 2002). Nevertheless, in this context, genetic processes form a state indicator according to the definitions now used within the framework of CBD and generally accepted by the Biodiversity Indicator Partnership (BIP – see http://www.bipnational.net/). A state indicator may also reflect the cause of indicator change and can therefore in some cases be also interpreted as an indicator of pressure.

The document on the pan-European C&I for sustainable forest management includes one indicator focusing on genetic diversity (FOREST EUROPE/UNECE/FAO, 2011). This indicator has been assessed with three verifiers:

- Area managed for *in situ* conservation.
- Area managed for *ex situ* conservation.
- Area managed for seed production.

This Indicator is a response indicator and apparently showed an increase in the area for genetic conservation and seed production in Europe in the 2011 report. However, the value of reporting only response indicators is limited, because they do not reveal the actual status of a given forest genetic resource itself. This would require range-wide monitoring of representative monitoring plots.

Evidently, in the context of genetic monitoring of conservation units of forest trees, we are concerned with state indicators, which are the focus of the present report.

Threats to forest genetic resources

Threats to forest genetic resources include risks regarding species, populations and genetic variability. These threats are both natural and human-caused (St. Clair and Howe, 2011). Large threats to forest genetic resources worldwide include habitat degradation and deforestation. Management practices may also have a negative impact on genetic resources if fast-growing trees with good phenotypes are selectively harvested while slow-growing trees with poor phenotypes are left as seed trees. Replacement of native stands with introduced tree species, or genetically distinct populations of native trees, can also lead to loss of genetic diversity. Climate change is not only a considerable threat to genetic diversity but it also enhances the threats from diseases, pathogens, insect attacks, fire and extreme weather. These factors are further complicated by the extensive and complex interactions taking place among these constituent factors (St. Clair and Howe, 2011).

The direct implications of climate change for forest genetic resources are not well understood, and moreover they largely depend on the actual climate changes that will occur. The Intergovernmental Panel on Climate Change (IPCC) has predicted that global temperature will rise about 1.8–4.0°C during the 21st century and up to 30% of the world's species will be at extinction risk (IPCC, 2013). For forest tree species, distribution models that are based on such scenarios predict considerable changes in tree species ranges in the next century. Whether or not tree populations will be able to respond and shift their distribution largely depends on their migration capacity and adaptive potential (e.g. Aitken *et al*., 2008).

Generally, postglacial migratory responses suggest a high potential for migration and long-term adaptation in forest trees. However, both climate and environment have changed substantially compared with the Holocene-era species expansion. It is therefore problematic to base future migration response expectations on extrapolation from past historical responses, especially as habitat loss and deforestation have led

to fragmentation and to the disruption of the natural pattern of gene flow. The potential for adaptation largely depends upon phenotypic variation, selection, fecundity, interspecific competition and biotic interactions. Forest trees have the advantage of exhibiting large phenotypic plasticity (defined as the capacity of individual plants to change phenotypes in response to changes in the environment) and high levels of genetic diversity, allowing for evolutionary adaptation to occur. In addition, populations of temperate tree species show clear clines in phenology and growth rhythm suggesting a capacity for rapid local adaptation (Aitken *et al*., 2008). At the same time, although widespread tree species with high fecundity are likely to persist and adapt, the long generation time of trees may put them at risk for mal-adaptation for a longer period. Epigenetic mechanisms for rapid adaptation to a changing climate may in some degree compensate for a delayed mal-adaptation. In Norway spruce, for instance, rapid genetic change in bud set from one generation to the next is probably related to an epigenetic memory in the progeny (e.g. Johnsen *et al*., 2005).

Which species and populations are more prone to experience the largest impact of climate change? The most vulnerable to climate change are probably species exhibiting rareness, very long generation time, limited phenotypic plasticity, low genetic variation and low dispersal abilities, as well as fragmented and disjunct species. The most vulnerable populations include populations at the rear edge of the species' natural distribution, populations with "nowhere to go", and populations threatened by habitat loss and diseases (St. Clair and Howe, 2011). Some indications of potential population extirpation in rear edge populations have been already inferred (Gimenez-Benavides *et al*., 2011).

Purpose of genetic monitoring

Genetic conservation programmes must take into account climate change and develop a system that will provide information on relevant changes in adaptive and neutral genetic variation through time in a species or a population. The purpose of genetic monitoring is exactly this. Genetic monitoring has been defined as the assessment of evolutionary potential and response of a species to temporal environmental change. Genetic monitoring can also be used to aid the maintenance of adaptive and neutral genetic variation by developing a warning system based on indicators and verifiers.

Based on more specific definitions, the aim of genetic monitoring is: (1) to assess the current status of genetic resources and quantify relevant changes in the light of preserving the long-term adaptive evolutionary potential of a species (Aravanopoulos, 2011); (2) to provide a practical framework for identifying adaptive evolutionary responses to environmental change (Hansen *et al*., 2012); or (3) to ob-

serve the dynamics of transition from the present to the future genetic status of a forest stand (Konnert *et al*., 2011).

Genetic monitoring encompasses something more than a study method, especially under changing environmental conditions. By observing temporal changes in populations, causal components can be inferred and their relative importance can be evaluated. Such an early detection mechanism would increase the chances of implementing management decisions that could mitigate potential harmful effects before irreversible damage occurs. Hence, genetic monitoring includes a prognostic value as well, to secure the conservation of processes that maintain genetic variation in natural populations (Aravanopoulos, 2011).

Challenges in genetic monitoring

The main challenges of genetic monitoring are to identify adaptive shifts or signatures of selection in populations, and to detect changes in the wealth and structure of genetic variation, as well as changes in effective population size. If such events are detected then the next major challenge of genetic monitoring is to identify the aetiology of these events. Very little is known about the actual environmental distribution of alleles at loci under selection, while high-quality models are yet to materialize regarding the environmental envelopes of adaptive variants that would address the potential effects of climate change. Under a genetic monitoring scheme, differentiation between selection and drift is important. We do expect directional selection to fix advantageous alleles, but this will not necessarily happen in a finite population because the effects of drift can mask the effect of selection if selection is weak or the population is small (Andrews, 2010). It is therefore important how we select the monitoring unit, especially concerning population size.

Investigating genome-wide variation, including loci under selection, may enhance the identification of selection and the detection of adaptive shifts. This is now achievable with next-generation sequencing (NGS). Despite the very high throughput capabilities of NGS, genome sequencing for a large number of individuals at the single species level has so far been rather limited and little used for forest trees (but see Holliday, Suren and Aitken, 2012). Recent developments in reduced-representation genome sequencing have brought direct sequencing closer to population genotyping. Methods such as restriction site-associated DNA tags (RAD-tags), and double digest restriction-associated DNA (ddRAD) sequencing (Davey *et al*., 2011; Hohenlohe *et al*., 2010; Peterson *et al*., 2012) are currently being established for several NGS platforms. Such methods will facilitate the acquisition of sequence information from thousands of loci, including loci under selection. Combined with other genetic information (such as outlier F_{ST} tests), signatures of selection may be detected

(Holliday, Suren and Aitken, 2012). A genome-wide analysis of loci under selection, as well as neutral loci, will lead to the broadest possible insight into the genetic processes manifested in the populations studied over time. Implementing state-of-the-art NGS approaches in genetic monitoring forms perhaps the main future technological and scientific challenge in genetic monitoring.

Approaches in genetic monitoring

In genetic monitoring, a number of indicators and verifiers have been proposed in various approaches. The number of verifiers reported in the literature ranges from 23 (Namkoong *et al*., 2002) to seven (Aravanopoulos, 2011). Table 1 shows a list of indicators and verifiers that have been proposed in various genetic monitoring schemes.

The most relevant genetic monitoring approaches regarding the genetic resources of forest trees have been developed by Namkoong *et al.* (1996, 2002), Aravanopoulos (2011) and Konnert *et al*. (2011). These are presented briefly below.

In the first comprehensive genetic monitoring system for forest trees, proposed by Namkoong *et al.* (1996), only one criterion was used: "conservation of the processes that maintain genetic variation". The criterion identified by Namkoong *et al*. (1996, 2002) was coupled with four indicators (levels of variation; directional changes in allele or genotype frequencies; migration among populations; and reproductive system) and a number of verifiers; in particular 18 verifiers suggested by Namkoong *et al*. (1996) and 23 suggested by Namkoong *et al*. (2002). The criterion and these indicators have been generally accepted as the goal and objects for measurement in most scientific and practical work dealing with conservation and management of forest genetic resources. The strength of the framework proposed by Namkoong *et al*. (1996, 2002) lies in its close tie to the basic genetic processes, which decide the adaptive evolutionary potential that is the overriding goal of forest genetic resources management.

For practical purposes, Aravanopoulos (2011) and Konnert *et al*. (2011) have adopted this framework. Aravanopoulos (2011) proposed a more simplified genetic monitoring system by suggesting that genetic monitoring should focus on the dynamic conservation units of forest trees and both key and rare or endangered species. The proposed genetic monitoring approach includes only three indicators (natural selection, genetic drift, and a gene flow-mating system) which are evaluated based on three demographic verifiers (age and size class distribution, reproductive fitness, and regeneration abundance) and four genetic verifiers (effective population size, allelic richness, latent

Table 1. List of indicators and verifiers from different sources

Indicators	Verifiers (genetic, demographic)	References
Levels of genetic variation	gene/genotype frequencies genotypic/allelic diversity	Allendorf *et al*., 2008 Bariteau, 2004 Granke *et al*., 2009 Graudal and Kjaer, 2006 Kuparinen and Merila, 2007 Laikre *et al*., 2008 McKinnell, 2002 Namkoong *et al.*, 1996, 2002 Schoen, Reichman and Ellstrand, 2008 Schwartz, Luikart and Waples, 2007 Geburek *et al.*, 2010 Konnert *et al*., 2011 Aravanopoulos, 2011 Hansen *et al*., 2012
Selection, gene migration genetic drift	gene flow population differentiation	
Gene flow	Effective population size (N_E) Sex ratio (dioecious species) Allele/genotype frequencies Genetic diversity parameters: allelic richness (A/L), N_A, P, H_E, H_O, latent genetic potential, F_{IS}, F_{ST} (+outlier tests) Inter-specific hybridization percentage (where applicable) Outcrossing or actual inbreeding rate	
Mating system	no. of effective pollen donors, latent genetic potential, no. of potential parents, significant traits in common-garden experiments	
Hybridization population structure population vital rates	detection of selective sweeps, neutrality of rates of evolution, temporal change of clinal patterns phenotypic frequency distribution, variation in phenological parameters, age class distribution, size class distribution, regeneration abundance, pollen dispersal, seed dispersal, physical isolation by distance, spatial aggregation of mating types, sex ratios, pollinator abundance, parental population density, proportion of filled seeds germination percentage, fertility, same age population differentiation, family structure, adaptively significant traits in common garden experiments	

genetic potential and outcrossing or actual inbreeding rate) (Aravanopoulos, 2011). This new scheme is a useful addition to the existing debate as it can make genetic monitoring more feasible and cost-effective in terms of field and laboratory work, but it does not solve all the problems (e.g. multiple indicators may still give conflicting results, and their treatment needs further development) and it still needs to be examined in applied conditions.

Konnert *et al*. (2011) have tested the German genetic monitoring system by using permanent monitoring plots. The study of Konnert *et al*. (2011) confirmed that the use of multiple indicators does not always provide a clear conclusion as to the functionality of the genetic system of a tree population. It also concluded that more research is needed to examine whether the four indicators should have the same or different weighting. Furthermore, the pilot field-testing confirmed the necessity and urgency for developing a genetic monitoring system, as problems in the genetic processes of tree populations are usually not immediately observable by measuring natural regeneration or vitality of seeds (Konnert *et al*., 2011).

DATABASES RELEVANT FOR GENETIC MONITORING

The establishment of a pan-European genetic monitoring system for forest trees creates two types of information needs. First, it is necessary to have information on forest areas that could be selected for genetic monitoring. Second, the data that will be generated through genetic monitoring have to be stored either in an existing database or in a new one developed for this purpose. Furthermore, if it is decided to collect and store plant material or DNA samples, or both, as part of genetic monitoring efforts, an additional database is needed to catalogue the sampled material. There are several existing databases in Europe that can meet these information needs and they are briefly discussed below.

EUFGIS Portal

As it has been agreed that the pan-European genetic monitoring system should focus on dynamic conservation units of forest trees, the EUFGIS Portal (http://portal.eufgis.org) is the most relevant database for identifying conservation units for genetic monitoring. It was developed as part of an EC-funded project (Establishment of a European Information System on Forest Genetic Resources, 2007–2011) to improve documentation and management of genetic conservation units of forest trees in Europe. The EUFGIS Portal is hosted by Bioversity International, and it is currently maintained as part of EUFORGEN activities.

The EUFGIS Portal provides geo-referenced information on the genetic conservation units based on 26 data standards at unit level (geographical area) and 18 data standards at population level (target tree species within a unit). However, the portal does not include any information on genetic diversity of tree populations occurring within the units. As of February 2015, the portal contained data on 3214 units, which are managed for genetic conservation of about 100 tree species in 34 countries. These units harbour 4061 tree populations. The data is provided and managed by national focal points nominated for this task by each participating country. So far, 36 countries have nominated their focal points.

Prior to creating the EUFGIS Portal, the project developed pan-European minimum requirements for the units in collaboration with EUFORGEN and a large group of experts in Europe (see Koskela *et al*., 2013). The units can be located in natural or man-made tree populations that are managed for maintaining evolutionary processes and adaptive potential across tree generations. Each unit should have a designated status and a management plan, and include one or more tree species recognized as target species for genetic conservation. The minimum sizes of the units should be 500, 50 or 15 reproducing individuals, depending on tree species and conservation objectives. Furthermore, silvicultural interventions should be allowed to enhance genetic processes of tree populations, and field inventories carried out every 5 or 10 years to monitor regeneration and the population size. However, the minimum requirements do not include any specification for genetic monitoring. The minimum requirements serve as a checklist for the national focal points, and only those units that meet them can be entered into the EUFGIS database.

ICP Forests

The International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests) was established in 1985 by the UN Economic Commission for Europe (UNECE) under its Convention on Long-range Transboundary Air Pollution (CLRTAP). The main objectives of ICP Forests is to monitor the spatial and temporal variation in forest condition at pan-European scale, gain better understanding of the impacts of natural and anthropogenic stress factors on forest ecosystems, and contribute to the formulation of forest policy at the national, regional and global levels (Lorenz, Fischer and Mues, 2005). ICP Forests created a systematic (16×16 km grid) large-scale monitoring network with approximately 6000 permanent plots (Level I), and an Intensive Forest Monitoring Programme with about 800 plots (Level II) covering the most important forest ecosystems in Europe. The Level I plots are used for annual crown and soil condition assessments and foliar surveys, while the Level II plots are used for a number of more specific analyses on the condition of the forests. The website of ICP Forest (http://icp-forests.net/) provides more information and an option to request ICP data for additional studies.

The ICP data has been used for monitoring changes in forest biodiversity, although not at the genetic level. To our knowledge, no genetic data is collected systematically from the ICP plots. However, the ICP plots offer an opportunity for genetic studies that focus on the impact of air pollution and climate change on genetic diversity in tree populations.

$(GD)^2$ database

The Geo-referenced Database on Genetic Diversity ($(GD)^2$) was created as part of the EVOLTREE project (Evolution of Trees as Drivers of Terrestrial Biodiversity, 2006–2010) and it is now maintained by INRA-Bordeaux. The database contains genetic and geo-referenced passport data on tree populations and single trees that have been sampled for genetic studies in Europe. It was developed to make available the genetic data sets (all types of markers) from published studies and it also provides a copy of the publication where a given data set was published. The database makes it possible to display the data in a standardized way and to carry out meta-analyses across species and geographical areas, for example. The $(GD)^2$ database can be accessed through the Quercus Portal (http://w3.pierroton.inra.fr/QuercusPortal).

DNA Repository Centre and EVOLTREE eLab system

The EVOLTREE project also created the DNA Repository Centre and the eLab system (virtual laboratory), which is a search engine through which it is possible to make integrated searches from 12 EVOLTREE databases on genetic and genomic resources. The DNA Repository Centre and the eLab system are managed by the Austrian Institute of Technology (AIT). The Repository Centre is a fully automated sample storage and data management facility. It currently contains about 645 000 samples from a network of 19 European laboratories that have carried out genetic studies on forest trees. More information on the DNA Repository Centre and the eLab system are available on the EVOLTREE website (www.evoltree.eu).

INDICATORS AND VERIFIERS

We, the members of the working group on genetic monitoring, debated for several months about the choice of indicators and the selection of verifiers. The state-of-the-art has been analysed in detail and the merits, advantages and disadvantages of indicators and verifiers reported in the literature (Table 1) have been evaluated. Furthermore, novel approaches have been exhaustively debated. Several factors have been considered in the final choice of indicators and verifiers, including, but not limited to: (1) the need for a restricted number of indicators suitable for pan-European application while not compromising essential genetic information needed; (2) the ease or difficulty of verifier assessment, taking into account the temporal nature of measurements; (3) technical expertise requirements; (4) financial considerations; and (5) indicator inter- or independence.

Proposed approach and associated indicators and verifiers

The proposed approach is built upon the conceptual framework of Namkoong *et al*. (1996) and the gene-ecological approach discussed by Graudal, Kjær and Canger (1995) and Aravanopoulos (2011), or implied in previous major studies related to the genetic monitoring of forest trees (e.g. Namkoong *et al*., 1996, 2002; McKinnell, 2002; Konnert *et al*., 2011).

The proposed scheme includes only two indicators, namely (I) selection, and (II) genetic variation and mating system, with a set of 10 or 11 verifiers (Table 2). Five verifiers are demographic quantitative parameters of straightforward estimation (six for dioecious species, where sex ratio will also be estimated), while the rest are population genetic parameters that require the use of genetic markers. Evidently, as genetic and genomic marker data will have to be generated, a battery of population genetic and genomic parameters can be easily estimated afterwards by applying specific population genetics and genomics software. In the present scheme, the minimum number of verifiers that can be used to assess genetic monitoring unit status in a comprehensive manner have been included.

Table 2. Proposed indicators and verifiers for the genetic monitoring of dynamic conservation units of forest trees in Europe, and proposed frequency of assessment (see text for explanation of verifiers and symbols)

Indicator	Metric Trait	Genetic Marker	Verifier	Annually or biennially	5 yr	10 yr
I – Selection	√		Age/size class distribution			•
	√		Mortality	•		
	√		Regeneration abundance		•	
			Fructification preferably annual, but at least in every visit to the unit; simple 4-scale system (core area)	•		
			Reproductive fitness in mast years (% of filled seeds and % germination) - optional			
II – Genetic variation and Mating System		√	Effective population size (N_E)			•
	√		Sex ratio (dioecious species)			•
		√	Allele/genotype frequencies			•
		√	Genetic diversity parameters: allelic richness (A/L), N_A, P, H_E, H_O, latent genetic potential, F_{IS}, F_{ST} (+outlier tests)			•
		√	Inter-specific hybridization percentage (where applicable)			•
		√	Outcrossing or actual inbreeding rate			

For the assessment of Indicator I – Selection – five metric verifiers are proposed:

1. Age and size class distribution, i.e. the proportionate representation of different age and size classes in a perennial plant population.

2. Mortality, i.e. number of trees that have died relative to the previous or a baseline assessment.

3. Reproductive fitness, i.e. the ability of an individual to survive and reproduce, evaluated as the combined percentage of filled seeds and germination (estimated based on the total number of seeds sampled and the total number of germinated filled seeds).

4. Regeneration abundance, defined as the number of seedlings per unit area.

5. Fructification, i.e. the total reproductive output in terms of fruit production.

For the assessment of Indicator II – Genetic variation and mating system – five population genetic verifiers (six for dioecious species) are proposed:

1. Effective population size (N_E), i.e. the number of individuals that will contribute genes to the next generation by means of cross breeding. Genetic marker estimation of N_E is preferable as it is very difficult to estimate N_E based on demographic

models. Genetic markers present the additional advantage of giving estimates that are more conservative.

2. Sex ratio, applicable to dioecious species, is usually estimated by phenotypic evaluation as the frequencies of male and female trees.

3. Allele and genotype frequencies, to detect allele frequency shifts and loss of alleles, as well as clinal variation changes by studying genetic monitoring units across the distribution range that may provide evidence for adaptive responses to environmental change.

4. Genetic diversity, the prerequisite for future adaptation and evolution, which is proposed to be evaluated by the following parameters: (1) allelic richness (A/L); (2) effective number of alleles (N_A); (3) percentage of polymorphic loci (P); (4) observed heterozygosity (H_O); (5) expected heterozygosity (H_E); (6) latent genetic potential, i.e. the difference between the total and the effective number of alleles summed over all loci; and (7) F-statistics, i.e., inbreeding coefficient (F_{IS}), coefficient of genetic differentiation among populations (F_{ST}) and outlier tests. It is noted that with large enough effective population size, reduction in genetic diversity parameters may reflect events of directional selection (Indicator I). In addition, F_{IS} and F_{ST} outlier tests will also give invaluable information regarding directional selection changes (Indicator I). F-statistics and heterozygosity analyses will also give insight into mating system consequences (see below, verifier 5).

5. Inter-specific hybridization percentage refers to the level of hybridization applicable when sympatric populations of cross-fertilizing species occur within a genetic monitoring unit.

6. Outcrossing or actual inbreeding rate (single locus and multilocus). Actual inbreeding rate is based on a combination of seed data (already available from Indicator I) and genetic markers. Outcrossing refers to the mating of genetically unrelated individuals and is the opposite of inbreeding.

This type of elaborate and comprehensive approach also raises issues regarding the advancement of specific procedural protocols, data management, database development and use, as well as intellectual property rights. Furthermore, rights and sharing regarding plant material, DNA samples, reference samples, data usage, results, and possible publications will have to be taken into account. Strong coordination, especially when selected units extend across pan-European environmental or geographical gradients, or both, is also needed. These issues have to be further analysed and clarified while developing

specific technical guidelines for monitoring purposes.

Proposed options for indicator and verifier assessment

Three types of data are associated with the assessment of the verifiers presented above: demographic, genetic and genomic. Based on these data types, three options for the assessment of a genetic monitoring unit are proposed, ranging from the most basic, inexpensive, rather incomplete but nevertheless indicative option, through to the most comprehensive, expensive, complete and state-of-the-art option. These options are:

First Option (Basic): Use of demographic data only. Indicator I is fully evaluated by five sets of verifiers. Indicator II is not evaluated, except in the case of dioecious species, where one demographic parameter (sex ratio) can be estimated. Nevertheless, this parameter is not enough to evaluate Indicator II as a whole.

Second Option (Standard): Use of demographic and genetic data. Indicator I is fully evaluated by five demographic sets of verifiers, and Indicator II is fully evaluated by five sets of genetic verifiers (six for dioecious species), assessed using SSR or SNP, or both, genotyping.

Third Option (State-of-the-art): Use of demographic and genomic data. Indicator I is fully evaluated by five demographic sets of verifiers and in addition by signatures of selection provided by the genome-wide analysis of sequence data. Indicator II is fully evaluated by five sets of genetic verifiers (six for dioecious species) based on genomic data (NGS-based data), providing greater accuracy and relevance in the estimates.

APPROACHES FOR IDENTIFYING POTENTIAL MONITORING REGIONS

The working group tested a number of different approaches for identifying potential monitoring regions at the pan-European level in order to develop the most appropriate approach for selecting genetic monitoring units among all units available in the EUFGIS database. Three approaches that were tested by the working group are presented below. Although these approaches, focusing on quantitative background data, have proven not to be suitable, they directly contributed to the development of the approach that was finally chosen. The approach finally selected is presented in Chapter "Criteria for the selection of the monitoring regions and the number of units per region" page 29.

Species distribution x environmental zone

This approach tested the environmental zone option (according to Metzger, *et al*., 2005; Metzger *et al*., 2013) for identifying potential monitoring regions in a way such that most environmental strata would be covered. This was done by overlaying the zone-level map and the species distribution map to obtain a breakdown of the distribution area size over the environmental zones. It was then tried to fit the distribution of the number of the units to the distribution of the area size over the strata. Although a number of problems were identified in this approach, the major problem, however, was that the available units do not cover the species distribution evenly, which resulted in very large gaps, even in the case of large, continuous species-distribution areas.

Species distribution x country x environmental zone x stratum

This approach attempted a more detailed and possibly more even distribution of potential genetic monitoring units, taking into account European environmental zones and strata. This was carried out by considering the ICP Forests Monitoring Network approach, which operates with 16×16 km grids. This option also showed reduced feasibility due to the uneven distribution of available units. For a number of different species, many partitions included a large number of units, while many other species were almost completely without units.

Species distribution × grid option

The main purpose of this third approach was to have a purely objective manner in which to select genetic monitoring units, based on a systematic selection system by overlaying grids with the species distribution. The grid option proved to require the use of different grid sizes (among, and even within, species), as the available units are very unevenly distributed within the distribution ranges of the respective species. It also proved very difficult to select units objectively along different gradients.

PRINCIPLES AND PROCESSES FOR SELECTING GENETIC MONITORING UNITS WITHIN MONITORING REGIONS

Accurate genetic monitoring of forest genetic resources requires, as with any other monitoring programmes, the most representative sampling possible, which could be simplified into the motto "the denser, the better". However, limited resources (personnel, funds, data handling capacity) impose the necessity for a more restricted coverage of units in the sampling. The goal of genetic monitoring is linked to the conservation of the long-term adaptive evolutionary potential. Therefore, when establishing acceptable limits to sampling, it is advisable to include all the ecological situations in which the species in question occurs. Other available information regarding genetic diversity and phylogeographical patterns, for example, must also be included in the decision-making process. Besides scientific reasons, the commitment of the different national administrations and agencies is paramount for the success of any genetic monitoring scheme. Hence, a genetic monitoring network (as a whole, including different model species) should involve all European countries and comprise a balanced selection of genetic monitoring units, taking into account the genecological criteria for selecting the genetic monitoring units (see also Chapter "Criteria for the selection of the monitoring regions and the number of units per regions").

Specific criteria to be taken into account in the selection of genetic monitoring units

Specific value

Genetic monitoring units must be representative of the genetic resources for which the monitoring region was selected.

Multipurpose units

It is advisable to concentrate monitoring efforts in multipurpose units. Preferably, a monitoring unit, besides being appropriate for genetic monitoring of one or more species, should as much as possible coincide with or be included in permanent plots, intensive study plots, or intensive study sites of national or regional forest inventories (IPC; other networks; plots already established in national or European projects; etc.). Several verifiers among those presented above are already measured in other routine inventories, or could be easily included.

Management criteria

In a large and dense European network of conservation units, many different types of diversely managed units are included, featuring different regeneration regimes as well as silvicultural and management techniques. It would be useful to assess the long-term influence of different large-scale management regimes on FGR, and, concurrently, to monitor the FGR available for (intensive) management. However, taking into account that the feasible number of units is likely to be limited for practical reasons, genetic monitoring units may focus on natural populations with minimal anthropogenic intervention. Additionally, the long-lasting commitment of owners and managers (and their formal agreement) to monitoring efforts will be needed, and should be emphasized. Evidently, it would be easier to achieve such commitment from owners and administrators of locations already appointed for conservation purposes. Therefore, although FGR are not confined to conserved areas and FGR outside these areas may play an important role and may interact with the ones in conserved areas, from a practical point of view, it can be reasonably expected that most (if not all) of the genetic monitoring units will be selected from existing conservation units.

Size

A minimum area of 4 ha has been considered as necessary for a genetic monitoring unit for stand-forming species, while for species with a scattered distribution the size will depend on the minimum required number of reproducing trees included in the plot.

Genetic monitoring unit

What is truly important is that genetic monitoring units must include a number of adult individuals of the species in question, numerous enough for an appropriate evaluation of its genetic resources. As an example, 30 unrelated individuals would be enough to achieve a 95% probability of detecting alleles with a frequency of 5% in the population. Sampling of 50 unrelated individuals would capture 99% of the additive genetic variation in the population. Since it can be difficult to assess the relationships among trees, a minimum number of 150 adult trees can be assumed as a reasonable proxy. The above value is also reasonable if we consider a minimum effective population size of 50 individuals, which may well correspond to a 3x census size (i.e. three times higher census population size). However, long-term genetic conservation requires larger populations; for example, 500–1000 adult trees would be needed to potentially capture low-

frequency alleles. Larger sizes allow the establishment of permanent monitoring plots following schemes such as the one used in the German system (Konnert *et al*., 2011). These figures can vary and be smaller when the genetic monitoring unit is located in marginal, or small but singular populations, or for scattered species.

Ownership

Practical experience has shown that usually it may be easier to achieve long-term commitments for genetic monitoring from a public administration, warranting that no important changes in land use and management regime will occur in the long term. For this reason, public properties may be generally preferred for the establishment of genetic monitoring units. As there are a variety of situations regarding ownership and its stability across Europe, the role of the EUFORGEN National Coordinators will be crucial during the selection process to ensure that units with a stable and predictable ownership are chosen.

Conservation status and threats

For a long-lasting programme, it will generally be advisable that monitoring units are not seriously threatened. In this sense, areas with legal conservation coverage may be considered as good candidates for the establishment of genetic monitoring units. Evidently it is also crucial that genetic monitoring work will be allowed within the units.

Genetic uniqueness

In certain cases, genetic uniqueness of the stand can make it relevant for monitoring purposes, and it can be included in the network although it is threatened. Specific actions should be taken in this case to counteract the menace in question.

DESIGN OF THE GENETIC MONITORING PLOTS

Genetic monitoring plots are established using both species-specific minimum numbers of individuals, and minimum sizes of the respective plots. The number of individuals in mature stands determines the size of the plot. Generally, a size of 4 ha is deemed adequate, as long as the above minima are met. Trees within the genetic monitoring unit must have reached reproductive age and natural regeneration must be evident in at least part of the area (Anonymous, 2006). There is a need for precise documentation in sampling activities, which makes it necessary to develop detailed sampling protocols suitable at the pan-European level. This will capitalize on the experiences of the German genetic monitoring system (Anonymous, 2006; Konnert *et al*., 2011) and the EC-funded FORGER project (http://www.fp7-forger.eu/).

In the process of selecting genetic monitoring units, high priority should be given to populations for which (in addition to the basic species-specific criteria for genetic monitoring unit selection) high data density and precise plot documentation is already available. In addition to the parameters in the EUFGIS database (26 data standards at the unit level and 18 data standards at the population level), the following tree parameters will be documented: tree identification label; tree coordinates; diameter at breast height (DBH) >7 cm; tree height; social ranking position; crown length and diameter; and quality traits (forking, top straightendness, epicormic shoots). Environmental data, if available from the vicinity of the plot (e.g. weather, atmospheric load, pollutant emission, soil, vegetation) should also be documented (Anonymous, 2006).

CRITERIA FOR THE SELECTION OF THE MONITORING REGIONS AND THE NUMBER OF UNITS PER REGION

The working group has agreed on the principles for identifying the genetic monitoring regions within which the genetic monitoring units will be selected. The approach used (based on the agreed principles) was fine-tuned by testing it with two selected keystone species, *Populus nigra* and *Fagus sylvatica*. Additionally, 13 species were also evaluated as a proof-of-principle exercise. Table 3 shows the list of species for which genetic monitoring regions have been identified and the potential number of genetic monitoring units proposed. While the working group has identified genetic monitoring regions and recommends the number of genetic monitoring units for each region, the final selection of genetic monitoring units will be the task of participating countries and their relevant authorities.

Moreover, the working group responded to the request of the Steering Committee to identify a subset of four to six species for which sufficient genetic data is already available and relevant genetic markers have already been developed. This subset will be used for preparing the ground for a pilot project and for initiating the pan-European genetic monitoring work before additional financial resources are secured. It was decided to select these species from the common pool of the "pilot" species used by the working group on the pan-European strategy for genetic conservation of forest trees (14 species) and the "keystone" and "endangered" species (15 species) used by the working group on genetic monitoring. There are 14 common species. The working group conducted a literature survey in order to identify published genetic information on the species that could be of interest and usable for genetic monitoring. The results of this survey of both published research papers and web-based genetic information resources are presented in Table 4.

Table 3. Keystone and endangered forest tree species investigated for the establishment of genetic monitoring regions

Keystone species
Abies alba
Castanea sativa
Fagus sylvatica
Fraxinus excelsior
Picea abies
Pinus brutia
Pinus cembra
Pinus halepensis
Pinus nigra
Pinus sylvestris
Populus nigra
Populus tremula
Quercus petraea
Sorbus torminalis
Endangered species at the pan-European level
Ulmus laevis

Table 4. Relevant genetic markers and associated genetic information on the 14 keystone species

Species	nSSR genetic markers and genetic information	SNP genetic markers and genomic information
Abies alba	Cremer *et al*., 2006; Gomory *et al*., 2012; Vendramin *et al*., 1999	Mosca *et al*., 2012a, b
Castanea sativa	Kremer *et al*., 2012	Marinoni *et al*., 2003
Fagus sylvatica	Jump, Hunt and Penuelas, 2007; Magri *et al*., 2006; Lander *et al*., 2011; Lefevre *et al*., 2012	Seifert, Vornam and Finkeldey, 2012
Fraxinus excelsior	Gerard *et al*., 2013; Heuertz *et al*., 2004	http://www.ashgenome.org/ ; http://oadb.tsl.ac.uk/
Picea abies	Scotti *et al*., 2002a, b; Tollefsrud *et al*., 2009	Chen *et al*., 2012a, b; Heuertz *et al*., 2006; http://bfw.ac.at/rz/bfwcms2.web?dok=9020
Pinus brutia	Keys *et al*., 2000	—
Pinus cembra	Salzer *et al*., 2009	Mosca *et al*., 2012a, b
Pinus halepensis	Chagné *et al*., 2004; Keys *et al*., 2000; Troupin, Nathan and Vendramin, 2006	Grivet *et al*., 2011
Pinus nigra	Gonzalez-Martinez *et al*., 2004	—
Pinus sylvestris	Garcia-Gil *et al*., 2009; Soranzo, Provan and Powell, 1998	García-Gil, Mikkonen and Savolainen, 2003; Garcia-Gil *et al*., 2009; Pyhäjärvi *et al*., 2007; http://bfw.ac.at/rz/bfwcms2.web?dok=9020
Quercus petraea	Neophytou *et al*., 2010	Vornam *et al*., 2011
Populus nigra	Cervera *et al*., 2001; Smulders *et al*., 2008a, b	Chu *et al*., 2009
Populus tremula	de Carvalho *et al*., 2010; Hall *et al*., 2007	Hall *et al*., 2007
Sorbus torminalis	Hoebee *et al*., 2007	—

For the selection of the subset of species, the working group considered: (1) species having both SSR and SNP information available; (2) species distributed in the broad ecological categories defined on the basis of geographical distribution (wide or restricted distribution) and ecological appearance (stand-forming or scattered); (3) species that present genetic conservation units with predominantly N>50 trees; (4) species that present genetic conservation units with populations characterized as marginal/scattered or rare/endangered, or both, but in any case with N>50; (5) species with a predominantly south-central and southern distribution, based on EUFORGEN distribution maps (http://www.euforgen.org/distribution_maps.html), which can be considered as having greater vulnerability to climatic change and therefore greater need for prioritization in genetic monitoring; and (6) species used by the relevant FORGER project. Based on these criteria, the working group proposes the following species: *Abies alba, Castanea sativa, Quercus petraea, Picea abies, Pinus halepensis* and *Populus nigra*.

It was decided to select the monitoring units from the conservation units entered into the EUFGIS database and, as much as

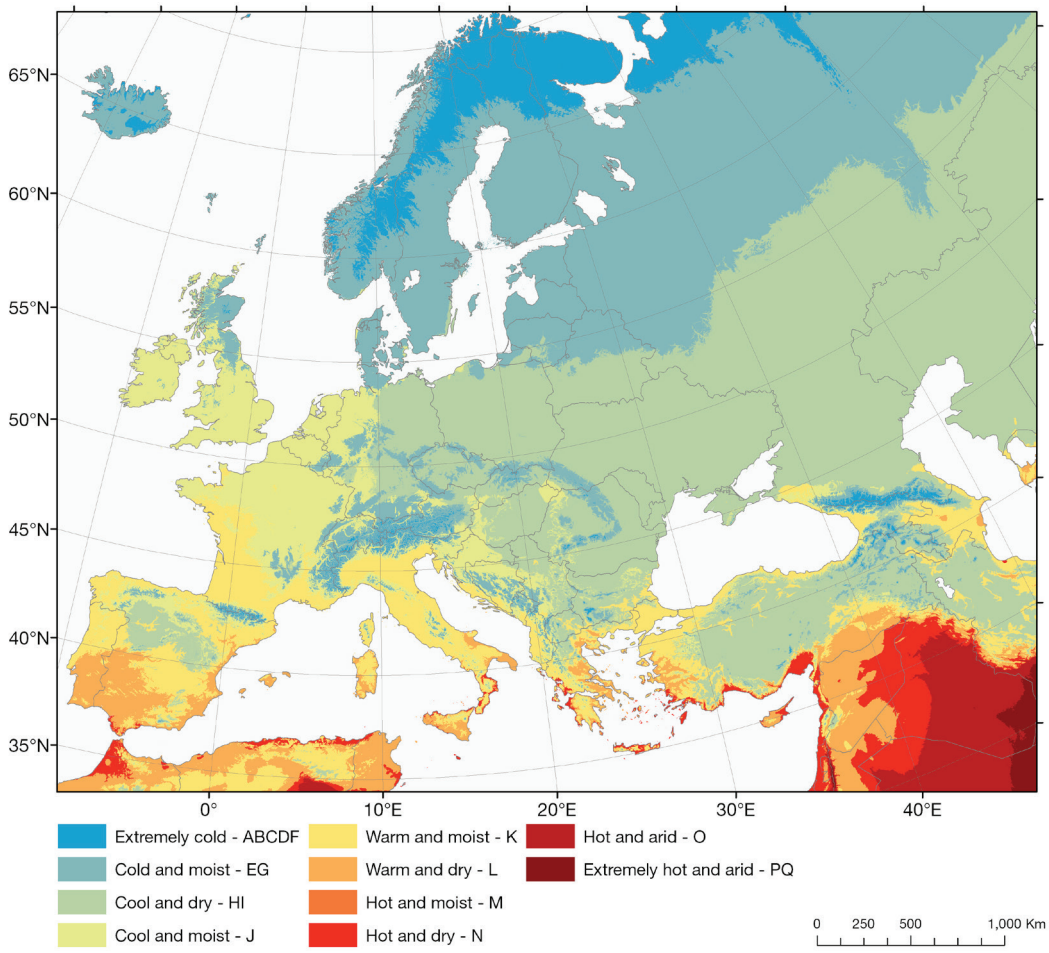

Figure 1. Aggregated environmental zoning of Europe (based on Metzger *et al.*, 2013) as developed by the EUFORGEN working group on the pan-European strategy for genetic conservation of forest trees (de Vries *et al.*, 2015).

possible, matching the units selected with the core network of dynamic conservation units identified by the EUFORGEN working group on the pan-European strategy for genetic conservation of forest trees. The minimum number of units per species will be equal to the number of the environmental zones within the species' range, as per Metzger *et al.* (2013) classifications. The maximum number of units per species will be equal to the number of country × zones of the same classification (Table 5). The working group developed its draft report based on the environmental zones of Europe as identified by Metzger, Leemans and Schroter (2005) and then prepared the final report based on the aggregated environmental zones developed by the other EUFORGEN working group based on Metzger *et al.* (2013) (Figure 1).

Table 5. Number of countries, environmental zones, and country × zones within the distribution range of selected species, and the number of potential monitoring units

Species	Countries[2]			Environmental zones			Country × env. zone		
	Total[3]	With units	Without units	Total[4]	With units	Without units	Total[5]	With units	Without units
Abies alba	20	14	6	5	5	0	69	31	38
Castanea sativa	25	5	20	7	3	4	84	8	76
Fagus sylvatica	31	19	12	5	5	0	102	39	63
Fraxinus excelsior	41	17	24	7	3	4	147	25	122
Picea abies	26	19	7	5	5	0	75	39	36
Pinus brutia	6	2	4	6	4	2	19	5	14
Pinus cembra	9	4	5	5	2	3	24	7	17
Pinus halepensis	5	3	2	6	3	3	18	4	14
Pinus nigra	15	12	3	7	4	3	61	23	38
Pinus sylvestris	33	17	16	6	4	2	97	33	64
Populus nigra	38	9	29	7	4	3	149	12	137
Populus tremula	41	5	36	6	3	3	140	6	134
Quercus petraea	36	23	13	7	4	3	125	34	91
Sorbus torminalis	32	10	22	7	4	4	124	13	111
Ulmus laevis	34	7	27	5	4	1	96	12	88

NOTES: (2) Of the 46 countries included in the pan-European region – see the list on page 5; (3) The countries were included when the occurrence of the species within the country × environmental zones exceeded the thresholds (>50 km^2 for species with restricted distribution, and 100 km^2 for widely distributed species); (4) Occurrence of the species within the environmental zones exceeding the threshold (>50 km^2 or 100 km^2). (5) Occurrence of the species within the country × environmental zones exceeding the threshold (>50 km^2 or 100 km^2).

The selection will be made according the following steps:

1. Tentative identification of monitoring regions is prepared on the distribution map of the species to identify rear edge and outlier populations.
2. The conservation units characterized by environmental zones are overlaid and additional units are identified.
3. Any available genetic marker data are overlaid in order to cover potential refugia and migration routes, i.e. covering as much of the species genetic diversity as possible.
4. Provenance trials indications are used to identify additional units.
5. The delineation of the identified monitoring areas is fine-tuned. Potential gaps in areas where genetic monitoring units are suggested, but where there are no conservation units, will be ascertained (additional units would need to be established and reported to the EUFGIS database).

The monitoring units will be selected following an expert-based approach, defining the total number needed for each species and the most appropriate placement within the species distribution range. For the exact identification of the genetic monitoring unit, the following additional criteria have been used:

- Population size: minimum 50 reproducing trees.
- Unit size: minimum 4 ha for stand-forming species.

The tentative results of the selection process for *Fagus sylvatica* and *Populus nigra* are presented in Figures 2 and 3, respectively.

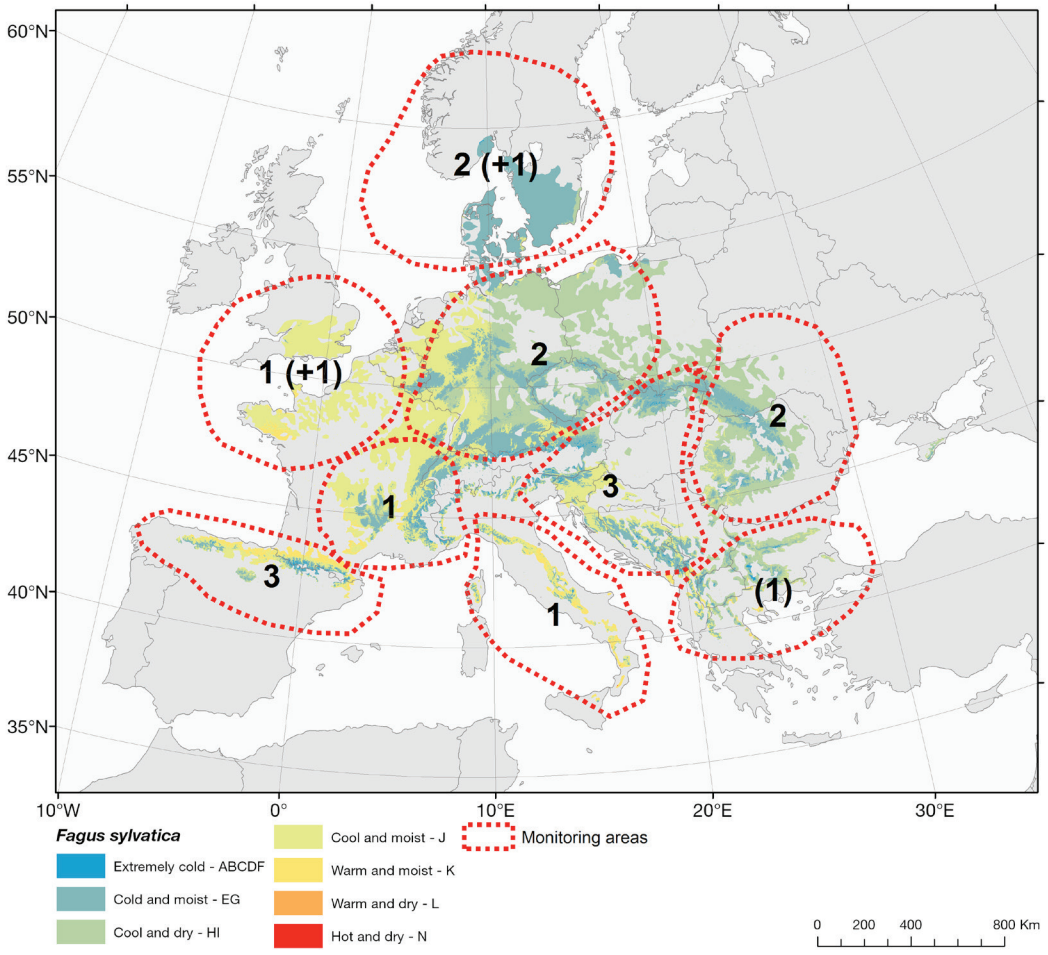

Figure 2. Monitoring areas (red circles) identified for European beech (*Fagus sylvatica*) and the number of units needed within each monitoring area (numbers in brackets indicate units that are not yet in the EUFGIS database).

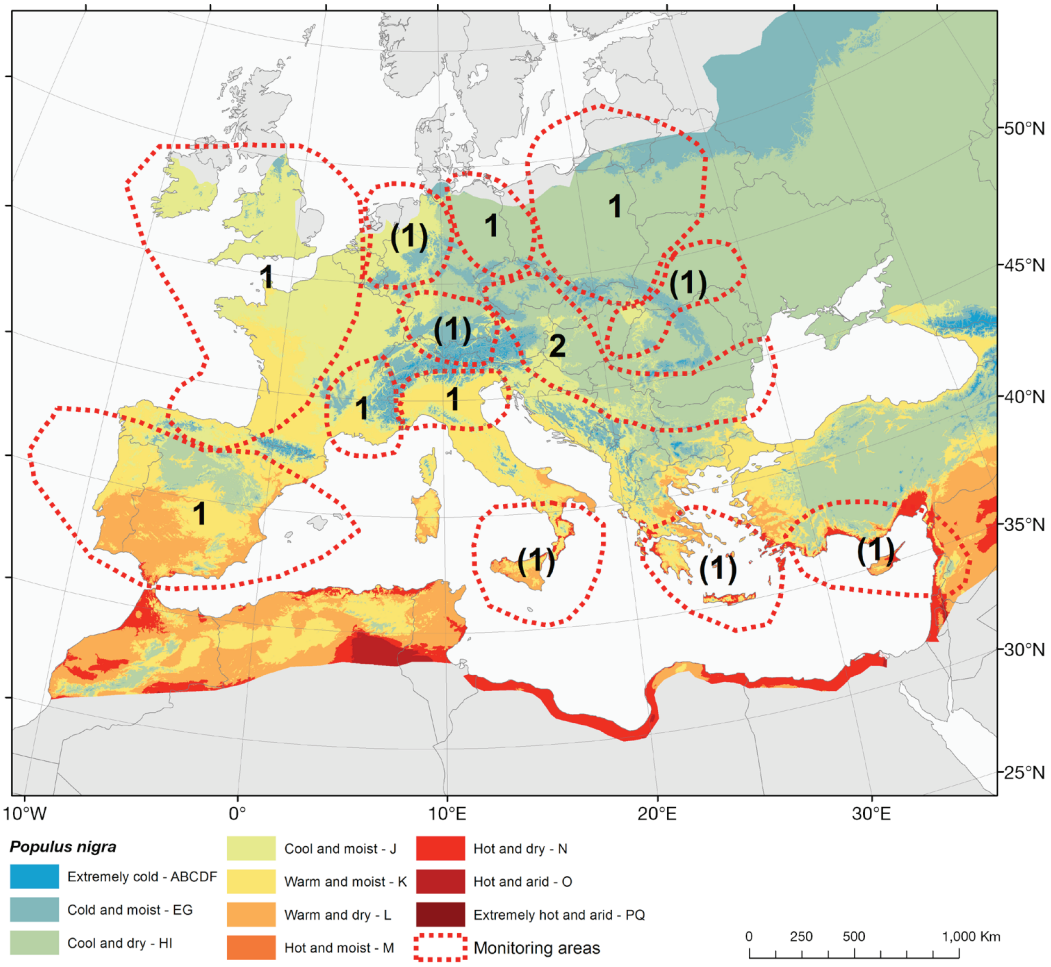

Figure 3. Monitoring areas (red circles) identified for black poplar (*Populus nigra*) and the number of units needed within each monitoring area (numbers in brackets indicate units that are not yet in the EUFGIS database).

COST OF GENETIC MONITORING

Sampling considerations in advance of cost estimation

Data or sample collection could be carried out in a single visit in the field. Sample sizes should at least be: number of individuals >30; number of loci >20; and number of seeds >1,000. The final number of neutral loci would depend on polymorphism levels (high polymorphism, i.e. ~5–20 alleles per locus, would be desirable), and should also be a function of the species chromosome number. A sequencing approach using NGS would probably gain much more than 1,000 markers genome wide. The number of seeds refers to the reproductive fitness assessment and could be decreased to number of seeds = 300 for the analysis of mating systems using neutral markers. The number of seeds that need to be analysed using genome-wide markers depends on the verifier in question. Preferably, the evaluation of a network of about 10 genetic monitoring units (populations) per species would provide enough resolution for assessing species and individual genetic monitoring unit status. A temporal frequency of one evaluation per decade has been provisionally proposed (for exceptions regarding verifiers requiring a more frequent assessment, see Table 2). This should be adequate given current levels of anthropogenic exploitation and environmental change.

Cost estimation

Fieldwork

Fieldwork needed for indicator evaluation would incur certain costs. The field sampling and Indicator I evaluation costs concern mainly labour. Labour costs vary considerably internationally. Therefore, it is proposed that an estimate of the time needed for these activities be presented as person-months, which could then be translated to actual labour costs on a case-by-case basis. For tentative purposes, the average hourly labour cost of €23.10 was used[1]. At the same time, costs for Indicator II pertain mainly to laboratory expenses, and they will therefore be presented as expenses per analysed data point.

Fieldwork refers to field tree measurements and sampling of leaves or buds, and seeds. It is estimated that six person-days per population (genetic monitoring unit) would be needed to account for verifiers 1, 3 and 4 of Indicator I, and verifier 6 of Indicator II (cumulative for 10-year evaluation). Measurements for

[1] Data from http://epp.eurostat.ec.europa.eu/statistics_explained/index.php/Wages_and_labour_costs

Indicator I (person-days per population) include seed extraction (2.0), estimation of filled seeds (1.5), planting (3.0), and monitoring germination and survival for six months (4). Therefore, a total of 165 person-days or about 8 person-months would be needed.

Traditional genotyping

Two obvious molecular marker choices for the estimation of Indicator II – Genetic variation and mating system – would be the highly variable multi-allelic microsatellites (SSRs) and the highly abundant bi-allelic SNPs. SNPs could be used currently for a few, very well studied species, where ample sequence information is already available. For the rest, contemporary evaluation would be probably based on SSRs. For some purposes, SNPs may be more advantageous than SSRs (Morin, Luikart and Wayne, 2004) depending on the number of SNPs and the level of variation revealed. They may also be present in both neutral loci and loci under selection. Thus, they may also be used to generate information on Indicator I, selection. For within genetic monitoring unit surveillance, the highly variable SSRs may be more advantageous, whereas SNPs may better reveal differences among the genetic monitoring units. Nevertheless, a very large body of SNP data should be generated, and problems such as ascertainment bias should be tackled before their everyday use in genetic monitoring. Large scale SSR or SNP analyses may be carried out through outsourcing where needed. Preferabely a standardized and optimized set of markers should be used to facilitate comparison between years and laboratories. The average cost of SSR analysis on a per-data-point basis, taking into account the literature, is about €0.40 at 2010 values (Aravanopoulos, 2011). Considering 10 populations, 30 individuals per population, 300 seeds per population and 20 SSR loci per individual, the cost on a per-species basis is about €26 500 for one evaluation per decade. This value does not include the cost for developing and optimizing SSR markers, which can be substantial, but probably decreasing due to next-generation sequencing (NGS) facilities. However, in many species these costs may be reduced as: (1) numerous SSR primers are already available and new ones continue to become available for a large number of key species; (2) successful application of SSR primers developed for one species has been reported in other species within a genus, or even for other genera within a family; and (3) if SSR primers have to be developed, this task will be carried out only once. It has been suggested that for bi-allelic SNPs, about four times more loci than SSRs are needed for reliable estimates of genomic variation and paternity analysis (Morin, Luikart and Wayne, 2004). By employing 80 SNP loci at about €0.05 per sample (Macdonald *et al.*, 2005), the corresponding cost per species for SNP analysis is about €17,000 assuming a success rate of an array being approximately 70%

(Vendramin[2], 2012, pers. comm.). Both SSR and SNP costs per sample continue to drop, as analytical tools become more cost efficient. According to the estimates presented above, genetic monitoring could be both feasible and cost-effective.

NGS methods

High cost, long laboratory procedures and bio-informatics challenges have so far limited a widespread adoption of NGS in population screening. However, the rapid advance of NGS technologies and the development of reasonably economical bench-top sequencing equipment means that genome-based sequencing could be widely adopted for population studies in the near future.

The cost for the different NGS options is highly variable, depending on the markers, methods and the platform used. For genetic monitoring purposes, the methods of choice must be specifically estimated depending on the available sequence information for the species and the resources already existing. Prices for NGS are constantly decreasing and Glenn (2011) concluded that, in 2010, Illumina had the broadest utility and lowest cost per read and Mb. The yield for Illumina MiSeq was then reported to be 1020 Mb/run with a cost of €0.57 per Mb. Cost estimates for construction of libraries, enzyme cutting and bar-coding (or tagging) of individuals to allow multiplexing should also be included. This may be a substantial expense, varying according to the platform used. According to Glenn (2011), the cost for an Illumina RNA-seq library may be about ten times the cost of the actual sequencing of the libraries. Detailed costs for different platforms and applications are presented in Glenn (2011). An example of a cost estimate of ddRAD tags is given in Peterson *et al*. (2012). For the discovery and genotyping of thousands of fixed differences in a laboratory cross and tens of thousands of SNPs in wild population samples, they report a cost of €15.50 per sample on the Illumina GAII platform. This includes about €4.00 for sample preparation and €11.50 for sequencing. For the Illumina HiSeq 2000 platform, cost is estimated to be well under €7.70 (about €4.0 for sample preparation and approximately €3.70 for sequencing). Furthermore, sample preparation prior to DNA extraction and DNA extraction must be included. The FORGER project (http://www.fp7-forger.eu/) operates with a cost of €1.00 per sample DNA isolation (Vendramin, 2012, pers. comm.). Cost estimates connected with data checking and handling, including data analysis, are not given, but should be carefully evaluated as well. This is a major expense, and research time needs to be included.

[2] Dr. G.G. Vendramin, Institute of Biosciences and BioResources (IBBR), 50019 Sesto Fiorentino (Firenze), Italy.

The choice of method – traditional genotyping with SSRs; SNPs; or NGS, preferably using a reduced representation sequencing approach – depends on what will be measured or monitored in a genetic monitoring framework. If the investigation of selection by demographic traits (Indicator I) is deemed adequate, then genetic markers are needed mainly for traditional population genetics parameters of genetic variation and mating systems (Indicator II). For this purpose genotyping, using about 20 SSR loci is sufficient. However, if a more detailed search for shifts in population adaptive potential is advanced, then markers for selection are also needed. In this case, reduced representation sequencing is one option that can be further explored.

Monitoring costs depend on: (1) the monitoring option employed (Chapter 'Indicators and verifiers' p.20); (2) options associated with the design of the genetic monitoring unit (Chapter 7); (3) the frequency of demographic evaluations and on the choice of molecular marker or system used; and (4) the level of analytical detail required. A special spreadsheet has been developed that accounts for the costs of all the different available options, choices and alternatives. A summary of the values indicated in this spreadsheet is presented in Table 6. As can be appreciated from Table 6, the evaluation of a stand-forming monoecious species based on the assessment of 10 genetic monitoring units during a 10-year cycle by using the standard option will cost approximately €115,000.

Cost for storage and infrastructure

Independently of the current method of choice, the temporal nature of genetic monitoring calls in most cases for the use of the same marker system for comparative purposes. Therefore, proper storage of plant material giving high quality DNA for utilization in the future is crucial. Proper storage will give the possibility for future a posteriori analysis of DNA by means that may not be available at present. DNA isolation techniques will improve and become inexpensive, while high DNA quality and quantity may for some applications be crucial. Therefore, plant material or DNA, or both, storage from the different genetic monitoring units should be properly organized and carefully evaluated. Currently, there are large facilities available for long-term storage of DNA, such as the DNA Repository Centre established by EVOLTREE. However, it could also be advisable to store plant material for the analysis of certain characteristics that could be lost during DNA isolation using current techniques. The proper methodology, facilities needed and costs (for both plant material and sample DNA storage, as well as for later access) should be considered.

Table 6. Estimated costs for one full cycle (one decade) of genetic monitoring in a monoecious stand-forming species assessed with 10 genetic monitoring units (as of 2014). Options refer to the genetic monitoring options presented on p.20. Calculations are based on the EU27 average of 8 hrs per day and €23.10 per hour ('Cost estimation' section of this chapter). Genetic analysis costs are based on values reported in recent literature ('Cost estimation' section of this chapter).

Indicators	1st Option - Basic			2nd Option - Standard					3rd Option – State of the Art		
	Fundamental[1]	Optional[2]	Total	Fundamental-I[3]	Optional	Total-I	Fundamental-II[4]	Total-II	Fundamental[5]	Optional[6]	Total
Indicator I (demography)	85,008	206,320	291,328	85,008	206,320	291,328	85,008	291,328	85,008	206 ,20	291,328
Indicator II (SSR data)	—	—	—	29,700	—	29,700	—	—	—	—	—
Indicator II (SNP data)	—	—	—	—	—	—	20,500	20,500	283,800	25,000	308,800
Total	85,008	206,320	291,328	114,708	206,320	321,028	105,008	311,828	368,808	231,320	600,128

Notes: (1) Refers to the estimation of demographic verifiers (Table 2), (2) Refers to further costs associated with plot establishment and maintenance (as per Konnert *et al.,* 2011), to the estimation of additional demographic data (as per Konnert *et al.,* 2011), or to the estimation of the originally proposed demographic parameters, but at a more frequent basis than that proposed in Table 2. (3) Refers to the estimation of demographic verifiers (Table 2) and the estimation of genetic verifiers based on SSR data. (4) Refers to the estimation of demographic verifiers (Table 2) and the estimation of genetic verifiers based on SNP data. (5) Refers to the estimation of demographic verifiers (Table 2) and the estimation of genetic verifiers based on next generation sequencing (NGS), in particular ddRAD-seq. (6) Refers to further costs associated with plot establishment and maintenance (as per Konnert *et al.,* 2011), to the estimation of additional demographic data (as per Konnert *et al.,* 2011), or to the estimation of the originally proposed demographic parameters, but more frequently than proposed in Table 2. In addition, this refers to the cost of a preliminary *de novo* genome assembly that would facilitate the NGS approach.

CONCLUSIONS AND RECOMMENDATIONS

The contemporary combination of evident climatic change and strong persisting adverse anthropogenic effects on natural ecosystems, and forests in particular, make the need for genetic monitoring paramount for the evaluation of forest genetic resources, whilst genetic monitoring providing an invaluable tool for future forest protection and sustainable management. It is a risk assessment method with prognostic value. Its conceptual focus is shifting from assessing the wealth of genetic variation and the processes that maintain genetic variation in natural populations, towards encompassing – in addition to the above – the evaluation and protection of their long-term adaptive potential. The approach presented above includes methods and protocols for the: (1) identification of genetic monitoring regions; (2) selection of genetic monitoring units within genetic monitoring regions, based on the European network of dynamic conservation units; (3) design of the genetic monitoring plot; and (4) selection of indicators and verifiers to be used. The application of genetic monitoring forms a comprehensive and unified scheme, unique for Europe and of global significance.

Specific recommendations for genetic monitoring of forest trees in Europe

The working group considers that genetic monitoring is achievable and advocates that genetic monitoring should be started as soon as possible. For this reason, the following are recommended.

- Implement and coordinate a genetic monitoring scheme at the pan-European level, at a minimum to include the model species listed in this Report. Until European funding is secured, national contributions are recommended to initiate the evaluation of demographic verifiers and sample collection. For the implementation of the proposed genetic monitoring scheme (see Section 'Species distribution × country × environmental zone × stratum' on p.21), external funding is necessary and should be sought, especially at the European level. It is noted that such a funding instrument should also allow for the participation of non-EU states (the so-called "third countries") that are EUFORGEN participants.

- Develop a manual or guidelines for the implementation of genetic monitoring at the pan-European level (including prioritization of species and tasks, fine-tuning of genetic monitoring unit selection, protocols, procedures, frequency of temporal assessments, description of the "ideal" genetic monitoring unit, suggestions on potential baseline values, establishment of critical levels of differences between temporal assessments, treatment of conflicting results, and detailed cost estimations).
- Facilitate the finalization of site selection, and coordinate early work in sample and data collection. In addition, identify climatic stations located in the proximity of selected sites and check pertinent data availability.
- Ensure that the EUFGIS database is maintained and further expanded, given its central role in the selection of genetic monitoring regions and genetic monitoring units.
- Identify facilities, such as the EVOLTREE DNA Repository Centre, that can be used for the long-term conservation of samples (plant material and DNA samples) and estimate relevant costs.
- Elaborate detailed estimates for minimum funding requirements for the different options as discussed in Section 'Species distribution × country × environmental zone × stratum' on p.21.
- If possible, acquire cost estimates of other monitoring programmes for comparative purposes.

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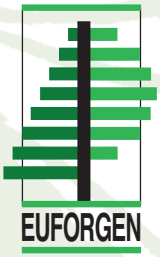
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